METHOD AND DEVICE FOR SCREENING MOLECULES IN CELLS

The present invention relates to a method and to a device for carrying out reactions on one or more cells or on cell tissues or cell networks or between cells.

The entry of molecules into the cell represents a key step in biotechnology. Most commonly, in order to study this step, the biological effects of a family of molecules must be screened in parallel, under the same conditions. The increasing amount of DNA sequences and of other molecules available to be tested on cells makes the use of automated methods, that are systematic in nature and have high yields, essential.

Document WO 01/07159 discloses a device for carrying out biochemical protocols in series in microreactors. However, this device does not make it possible to work on cells.

Document WO 95/34374 describes a device and a method for carrying out microreactions in series. However, this device and this method do not envisage the performing of "transfection" reactions in which a reagent penetrates into a cell. In fact, the cell is a heterogeneous living organism, the survival and the reaction of which require specific conditions, in particular as regards gas exchanges. These parameters are not taken into account or even envisioned in that document.

At the current time, most of the methods for screening molecules do not make it possible to use living cells; they are carried out with binomes of isolated molecules. In the case of screenings involving living cells, the screened molecules rarely penetrate into the cells, they recognize only surface captors, such as for example receptors.

For example, the transfection of cells with DNA families is currently carried out in dishes comprising wells distributed in the form of matrices. This method has the drawback of consuming considerable amounts of reagent, and of requiring laborious devices for detecting the molecular interactions. In addition, the systems for analyzing fluorescence in wells have the drawback of being large in size, the well-containing plates' own fluorescence must be taken into account, and the analysis must be carried out well by well, without any overall vision of the device as a whole.

J. Ziauddin *et al.*, Nature, 411, 107-110, 2001, reports an automated method of transfection: DNA is deposited in the form of a dispersion in gelatin in a matricial manner on a glass slide. After drying, the positions comprising the DNA are treated with a lipid transfection agent and the plate is then placed in a medium into which are dispensed cells. On the glass slide, the gelatinized DNA is present in solid form and the transfection takes place in semi-solid phase by binding the DNA molecules to lipids promoting penetration of the DNA into the cells adjacent to the DNA deposits. A matrix of transfected cells at the positions corresponding to the DNA deposits is obtained. However, this method has the drawback of being relatively imprecise and nonreproducible. The attachment via the gelatin does not make it possible to control the detachment of the transfected DNA. Neither does it make it possible to improve the transfection efficiency. It is difficult to obtain expression or the blocking of expression of a sufficient amount of protein by this method. In addition, only one cell type can be used for each glass slide.

There remains therefore the need for a method of transfection, and more generally a method of reacting compounds with biological cells, it being possible for

said method to be automated, using minimal amounts of reagents and giving reproducible results. In addition, it is desirable to be able to carry out several reactions in series in the same cell, to be able to work on complex cell systems (two types of different cells), on cell tissues or on cell networks, and to place several cell systems in parallel. It is also desirable for it to be possible for the genome of the cells used to be modified beforehand in order to prepare the detection of the molecular interactions, for example by introducing fluorescent protein genes.

A subject of the invention is therefore a method for reacting a reagent R with at least one cell C, said method being characterized in that:

- the cell C is deposited on a support S comprising a substantially flat surface, in the form of an aqueous drop on said surface;

- the substantially flat surface of the support S on which the aqueous drop containing the cell C has been deposited is covered with a separation film F, allowing gases to pass through and preventing evaporation of the aqueous drops deposited on the support S, F being non-miscible with the reagent R;

- the reaction between the reagent R and the cell C is triggered by introducing the reagent R into the aqueous drop containing the cell C. Several variants exist for the introduction of the reagent R into the cell C:

  - According to a first variant, an aqueous drop containing the cell C is deposited on the support S, a second aqueous drop containing the reagent R is injected, using any appropriate injection means, directly into the drop containing the cell C. Such a variant is illustrated in figure 1.

- According to a second variant, a first aqueous drop is deposited on the support S and then a second aqueous drop is deposited on the same support in the vicinity of the first; one of these drops contains the cell C, the other the reagent R, and the reaction of the reagent R with the cell C and, optionally, its transfection into the cell C is triggered by the fusion of the two drops. The displacement and the fusion of the drops can be obtained by vibration within the support, by electrophoretic displacement of the electrically charged drops or by mechanical or optical tweezers. They can also be obtained by modification of the surface properties of the support under the effect of an electric or magnetic field, or by optical or thermal treatment. Such a variant is illustrated in Figure 2.

- According to a third variant, the reagent R is attached to the support S or to the film F, the cell C is deposited, in the form of an aqueous drop, on the support S and the reagent R is then detached from the support S or from the film F in order to allow it to react with the cell and, optionally, to allow it to be transfected into the cell. This variant is illustrated in Figures 3 and 7.

In the present invention, the term "transfection" is used to denote the penetration of a molecule of a reagent, whatever it is, into a cell.

A subject of the invention is also a device for reacting a reagent R with a cell C, this device being characterized in that it comprises:

- a support S comprising a substantially flat surface covered with a separation film F allowing gases to pass through and preventing the evaporation of the aqueous drops deposited on the support S, F being

non-miscible with the reagent R,

- means for depositing, on said surface and under the film F, aqueous drops containing the cell C,

- a controlled-atmosphere chamber in which the support S is placed so as to allow the cell C to survive.

Preferably, the support S consists of a plate which may be made of silicon, of glass or of polymer, such as for example of polyurethane, of nylon, of polyester, of polyethylene, of polypropylene, of polyfluorocarbon, of poly(methyl methacrylate) (PMMA), of polycarbonate, of polyvinyl chloride (PVC), of polydimethylsiloxane (PDMS) or of polysulfone.

According to the invention, the drops are attached to the support by capillarity, by means of surface tension forces. Preferably, the support S has a substantially flat surface comprising at least one means intended for the reception of the aqueous drops.

Preferably, the means intended for the reception of the aqueous drops consists of zones of the substantially flat surface of the support S that range from 5 μm^2 to 5 mm^2 in size.

According to a first variant, it may be envisioned that the support S exhibits on its flat surface a hydrophobic nature, and comprises one or more hydrophilic zones constituting said reception means. According to another variant, it can also be envisioned that the support S comprises, on its flat surface, cavities ranging from 1 micron to 1 millimeter in depth and constituting said reception means. It can also be envisioned that the support S is a plate equipped with outgrowths of small thickness, from 1 micron to 1 millimeter, arranged on its surface and intended to promote the attachment of the drops. Finally, it can be

envisioned that the support S is a plate equipped with at least one wire, to which the drops attach. The depositing of two drops on the same reception means will promote the fusion of these two drops and therefore the reaction of the reagent R with the cell C. Preferably, the support S exhibits on its flat surface a hydrophobic nature, and comprises one or more hydrophilic zones constituting the reception means. In order to confer on the flat surface of the support a hydrophobic nature, said surface is preferably covered with a hydrophobic material such as a polyfluorocarbon, for instance polytetrafluoroethylene or Teflon®, with a silane, for instance perfluorosilane. The hydrophobic zone of the support can consist of a surface structuring that is indented on a nanometric scale, such as the "black silicon" used in optics. Examples of commercial slides of this type are the superteflon 40-well D2 mm immunofluorescence slides sold by the company MERCK EUROLAB division POLYLABO. Even more preferably, the support also comprises a second means for receiving the drops, superimposed on the first, such as, for example, a hydrophobic flat surface and hydrophilic outgrowths of small thickness, or a hydrophobic flat surface and hydrophilic wells, or a hydrophobic flat surface and a hydrophilic wire.

According to a variant of the invention, the support has a surface whose hydrophilicity/hydrophobicity properties can vary under the influence of a parameter such as temperature, an electric field, a magnetic field or an irradiation. The support may thus be active, so as to cause the drops on its surface to change, using the principles of droplet microfluidics. This amounts to dynamically modifying the surface properties of the support (for example the variations in surface tension/energy) so as to cause the drops to move in a controlled manner. Thus, the drops of cell cultures can go through various reaction steps carried out within the support: it is possible to fuse two

drops which are close together (one with the reagent and another with the cells, for example).

To produce this type of support, Shenderov *et al.* ("*Electrowetting-based actuation of liquid droplets for microfluidic applications*", Applied Physics Letters, vol. 77, No. 11, p. 1725-1726, September 2000) describes the use of the modification of the surface energies of a hydrophobic layer when an electric field is applied: the surface tension decreases with the intensity of the field, the surface becomes less hydrophobic, or even hydrophilic. The control and the movement of the electric field make it possible to displace the drops of liquid on this surface. This method was patented by the company Nanolytics (Shenderov *et al.* "Actuators for microfluidics without moving parts", No. US 6,565,727; 2003), but without the use of cell cultures.

Another manner in which these surface properties can be modified consists of the physicochemical modification of the surface layer of the support, still using an electric potential. For example, the change in conformation of an SAM layer ("self-assembled monolayer", for example modified thiols, comprising at least one hydrophilic end and one hydrophobic chain), demonstrated by Lahann et al. ("*A reversibly switching surface*", Science, vol. 299, p. 371-374, January 2003), makes it possible to go from a straight conformation of the molecules within the surface layer, which then is hydrophilic in nature, to a curved conformation, in which it is hydrophobic in nature.

Similarly, the temperature can be used as a means of changing the surface properties of a support. Liang *et al.* ("*Preparation of Composite-Crosslinked Poly(N-Isopropylacrylamide) Gel Layer and Characteristics of Reverse Hydrophilic-Hydrophobic Surface*" Journal of Applied Polymer Science 72:1, 1999) describes a polymer

which is hydrophilic at low temperatures (<30°C) and hydrophobic above this. By integrating a system of localized control of the temperature under the substrate, it is possible to control the surface properties.

It is also possible to set up a support for which the properties of the surface layer change according to whether or not light is applied (electromagnetic fields). Ichimura *et al.* ("*Light-driven motion of liquids on a photoresponsive surface*", Science, vol. 288, p. 1624-1626, June 2000) describes such a surface: a layer of polymer (calyx[4]resorcinarene), the terminal group of which (azobenzene) can change isomeric conformation after asymmetric photo-irradiation. When these cyclic groups in the *trans* conformation (hydrophilic layer) are exposed to UV radiation (365 nm), they change to the *cis* conformation (hydrophobic). The reaction is reversible using blue light (436 nm). By selectively and gradually lighting the polymer layer, it is possible to displace liquid drops in a controlled manner.

According to a variant of the invention, the reagent R is attached to the support S before depositing of the aqueous drop containing the cell C. Such devices are known to those skilled in the art for other uses: they are DNA chips as described by:

- Eisen M.B., Spellman P.T., Brown P.O., Botstein D. Cluster analysis and display of genome-wide expression patterns, *Proc Natl Acad Sci USA*. 1998 Dec. 8; 95(25): 14863-8;
- Haab B.B., Dunham M.J., Brown P.O., Protein microarrays for highly parallel detecting and quantitation of specific proteins and antibodies in complex solutions, *Genome Biol.* 2001 Jan 22; 2(2): RESEARCH 0004.1-0004.13;
- Livache T., Bazin H., Caillat P., Roget A., Electroconducting polymers for the construc-

tion of DNA or peptide arrays on silicon chips, *Biosens Bioelectron.* 1998 Sep 15; 13(6): 629-34.

The same principle can be applied to molecules other than polynucleotides. Molecule chips are described in: Kuruvilla *et al.*, Glucose signalling with small molecule microarrays, *Nature* (2002), 416 p. 653. In all cases, the reagent molecule is first attached to the chip (for example by covalent attachment to a glass slide). According to the present invention, the molecule may optionally be detached after depositing of the aqueous drops containing cells on the molecule chip.

The detachment of the reagent molecule can be carried out in a known manner by one of the following means:

- UV-photocleavage using a site for binding of the reagent to the support which is photocleavable, as illustrated in figure 5;

and when the reagent is a polynucleotide only:

- cleavage of the double-stranded DNA with restriction enzymes, or with other nucleases,

- modification of the hybridization stringency: a change in salt concentration, in temperature or in redox conditions of the medium makes it possible to separate two DNA strands.

In certain cases, it is envisioned that the reagent R remains attached to the substrate.

According to the invention, the substantially flat surface of the support S is covered with a separation film which fulfills three functions:

- it prevents unwanted fusion of the aqueous drops,

- it prevents evaporation of the aqueous drops deposited on the support,

- it allows gases to pass through, in particular O_2 and CO_2, the latter two functions being intended to enable the cells to survive in their drops.

The film F may be of various natures:

- it may be a non-water-miscible liquid such as, for example, an oil. Up until now, it was known how to use oil to preserve certain cells; however, it had never been used to carry out reactions on cells. Among the oils that can be used in the method and the device according to the invention, mention may in particular be made of mineral oils and silicone oils. It was also possible to use, as liquid L, an organic solvent that is non-miscible with the compounds to be treated (cells and reagents), such as, for example, octane. A light mineral oil is preferably used;

- it may also be a gas such as air saturated with moisture;

- it may subsequently be a flexible, solid film, such as a PDMS or polydimethylsiloxane film or a nitrocellulose film;

- it may, finally, be a rigid honeycombed cover made of porous material, the size of the cavities being adjusted so as to be able to contain the drop of cell(s) and, optionally, of reagent. According to a variant of the invention, the rigid honeycombed cover may be functionalized, in each cavity, with a molecule of reagent and may thus constitute a molecule chip or a nucleotide chip said to come into

contact with the support on which drops of cells have been deposited in a manner that is symmetric with respect to the cavities. This variant of the invention is illustrated in figure 7.

When the separation film is a gas or a liquid, the aqueous drops containing one or more cells or a reagent are advantageously deposited on the support S and under the separation film by means of fine capillaries, as illustrated in figure 1. Preferably, these capillaries are connected to a pump or syringe pump making it possible to control the volume of the drops.

The reagents or the cells can also be dispensed by means of a conventional system such as those used for the production of DNA chips. Mention may, for example, be made of piezoelectric systems for compressing a cavity and ejecting a drop via a nozzle. Reference may be made, on this subject, to N. Takada *et al.*, Proceeding of the SID, vol. 27/1, **1986**, 31-35.

Preferably, the ejected drops pass through the liquid or gas film by virtue of their rate of ejection and/or by gravity, this liquid or this gas being lighter than the solution to be deposited. When the separation film is a solid film or a rigid cover, it is deposited on the support, after depositing of the aqueous drops of cells and, optionally, of reagents, by the same means as described above.

The displacement and the fusion of the drops can be obtained by vibration within the support, by electrophoretic or electromagnetic displacement of the electrically charged drops or by mechanical or optical tweezers. It can also be obtained by a modification of the surface properties of the support brought about by the application of an electrical magnetic field or a thermal or optical treatment.

Preferably, the support S of the device is mobile, so as to allow it to move from a first depositing means to a second depositing means, and optionally to other depositing means. The support S may, in certain cases, consist of a solid film attached to rollers at its two ends, the rollers being equipped with winding means so as to allow displacement of the film and therefore displacement of the drops which have been deposited on it.

Generally, the method according to the invention envisions the displacement of the support S after the depositing on the support S of the first series of drops, whether it is drops of cells or drops of reagent.

According to the invention, the support S is placed in a controlled-atmosphere chamber, the temperature, the hygrometry and the CO_2 content of which are adjusted so as to allow the cells to survive.

Such devices are in particular controlled-atmosphere incubators. The temperature in such a device can range from 35 to 42°C, a preferred temperature being between 36.5 and 37.5°C. The temperature variation may in particular be used to induce cell differentiation.

The CO_2 level is preferably maintained at between 3 and 5%. The oxygen O_2 level is preferably that of ambient air.

For example, it is possible to envision maintaining the cells in aqueous drops on the support S in an incubator at 37°C, with 95% air, 5% CO_2 and 97% humidity.

It is also possible to envision that the entire reaction device: support, separation film, depositing means, detection means, etc, is placed in the controlled-atmosphere chamber.

It is also possible to envision that only the supports on which have been deposited the drops of cells and the separation film are placed in a controlled-atmosphere chamber.

Advantageously, it is possible to envision that the aqueous drops containing one or more cells, a cell tissue or a cell network comprise a culture medium.

In fact, the establishment of cell cultures depends on the ability of the cells to maintain their proliferation and therefore on the conditions essential to their growth.

Advantageously, it is envisioned that the aqueous drops of cells comprise MEM, or minimal essential medium, sold by the company GIBCO BRL under the cat. reference No. 12000-022.

The culture medium may also contain other constituents, such as calf serum, or one or more antibiotics intended to control the sterility of the medium, for instance penicillin.

It is also possible to envision using, in the culture medium, chemical agents which induce differentiation of the cells, for instance bromodeoxyuridine.

It is also possible to envision that the aqueous drops in which the cells C are in culture are gelled, using any known gelling agent, for instance agar or gelatin.

Advantageously, the aqueous drops containing the cell(s) or the cell tissue or the cell network, and/or the aqueous drops containing the reagent, comprise one or more constituents intended to promote transfection, for instance liposomes. Such transfection agents are described in particular in documents WO 01/20015 and WO 98/33932.

Other means intended to promote transfection can also be used in the device of the invention, such as: electroporation or microprecipitation. These methods of transfection, well known to those skilled in the art, are described in particular on http://opbs.okstate.edu/~melcher/MG/MGW4/MG43.html.

According to the invention, it is also possible to envision that the device comprises:

- means for supplying energy to one or more drops deposited on the support;

- means for optical treatment of one or more drops deposited on the support;

- means for applying a magnetic field or an electric field to one or more drops deposited on the support, in particular for allowing electroporation;

- means of detection focused on one or more drops deposited on the support.

The means used in the devices according to the invention will preferably be connected to a control device making it possible to automate the device and the method according to the invention.

Among the means for supplying energy, mention may in particular be made of means of thermal treatment, which can consist, for example, of a heating device which can be placed in the vicinity of the support S or attached to this support, and which is intended to bring the drops to an appropriate temperature. For example, the heating means may consist of electrically conducting wires that also serve as a means for receiving the drops.

The detection means are in particular devices intended

to measure the fluorescence or the radioactivity of one or more drops or of the cells contained in one or more drops.

The optical treatment means are in particular means for treatment with ultraviolet rays, the latter being known to induce crosslinking between complementary strands of DNA and between DNA and proteins.

The use of the device and/or of the method according to the invention has many advantages: very small amounts of materials can be used: a single cell per drop makes it possible to carry out a transfection experiment. It is possible to work with very small drop volumes, of less than 1 microliter, preferably of 0.1 to 1000 nanoliters containing 1 to 500 cells, even more preferably of 0.1 to 10 nanoliters containing 1 to 10 cells. Advantageously, drops containing from 1 to 100 cells are used. It is also possible to envision working on larger volumes, in particular greater than a microliter (10 to 100 µl, containing 500 to 100 000 cells). This method also makes it possible to use small amounts of reagent. The separation film F makes it possible to control the gas exchanges of the culture medium of the cell and its sterility. It also makes it possible to separate drops that are not intended to react together. Finally, this method makes it possible to improve the transfection efficiency: each cell used in the method can be transfected. The cell cultures in the form of drops under the separation film can be conserved for at least 24 hours and for up to several days without notable modifications of their cellular activity being observed (without notable influence on the proliferation and growth of the cells).

The method and the device according to the invention also make it possible to carry out batteries of reactions:

Several aqueous drops each comprising at least one cell can be deposited onto the support S, said drops being isolated from one another. Preferably, each of these drops is placed in a different reception means. All the cells may be identical, but it is also possible to envision placing different cells (at least two sorts of different cells) in the various drops. Drops containing the reagent(s) are deposited, in the vicinity of each drop containing a cell, so as to allow the fusion of one drop containing the appropriate reagent with the drop containing the targeted cell. For carrying out batteries of reactions, a support comprising reception means arranged evenly in the form of matrices is advantageously envisioned, so as to allow the method to be automated.

Advantageously, the support and the capillaries intended to deposit the aqueous drops of cells and of reagents are connected to control means so as to allow the method to be automated.

The method and the device according to the invention therefore make it possible to carry out simultaneously and in an automated manner a large number of reactions of a reagent on a cell, varying the nature of the reagent and of the cell, while at the same time working on extremely small volumes.

Among the cells that it may be advantageous to study by means of this method, mention may in particular be made of:

- primary cells,
- hybridomas,
- cell lines: the cells can perpetuate endlessly and thus form lines,

- stem cells: they are obtained from a sample taken from an animal or from biopsies,

- a piece of cell tissue (the cells are not individualized),

- mixtures of the various type of cells stated above.

The cells are cultured in culture medium (aqueous) in a known manner. It is also possible to culture heterogeneous cells for several days and to use this mixture.

According to a variant of the invention, when all the cells to be reacted on the same support are identical, the procedure may be carried out in the following way: the support is a hydrophobic plate comprising hydrophilic zones, it is immersed in an aqueous solution containing the cells, and then it is removed from this solution, allowing the excess liquid to run off. The drops of the medium containing the cells are retained in the hydrophilic zones. This step is followed by the depositing of a layer of separation film F and by the depositing of the drops containing a reagent or other cells. According to the nature of the film F (fluid or solid), it is deposited before or after the depositing of the drops of reagent or of other cells.

Among the reagents R which can be used in the method and the device according to the invention, mention may be made of:

Chemical molecules of all natures, in particular natural organic molecules, molecules derived from organic synthesis and from combinatorial synthesis, molecules extracted from biological samples and molecules extracted from biological samples, that have been modified by synthesis. Mention may in particular be made of polynucleotides: single-stranded and double-stranded RNA molecules, in particular siRNA (small

interference RNA) molecules; single-stranded and double-stranded DNA molecules; PNA (peptide nucleic acid) molecules which are peptide-nucleic acid chimeras; ribozymes; double-stranded interfering RNAs or proteins and peptides. Among the proteins, mention may be made most particularly of transcription factors.

The reagent molecules can be formulated in a solution ready to be deposited. They can also be prepared directly after depositing on the support, for example by synthesis, in particular organic synthesis, *in situ*, or by *in vitro* transcription in the drop. Prion-type molecules can also be obtained as drops by peptide polymerase chain reaction or PCR before transfection thereof into the cells. When nucleic acid molecules are used, they can be prepared by nucleic PCR. As has already been disclosed above, the reagent can also be attached to the support.

When DNA is used as reagent, it is advantageously in precipitated form. Calcium phosphate can, for example, be used, in a known manner. The DNA precipitation can also be carried out in the aqueous drop deposited on the support, by fusion with a drop of the appropriate reagent.

According to a variant of the device and of the method according to the invention, it can be envisioned to make several successive deposits intended to be fused:

It can be envisioned to successively deposit several reagents intended to transfect the same cell, and to observe their cumulative effects;

It can also be envisioned to deposit several drops of cells and to cause them to fuse, so as to reconstitute a cellular network of identical or different cells in order to be as close as possible to conditions encountered *in vivo*. For example, it is possible to

reconstitute networks of neurons on a scale of a few cells, by means of glial cells encountering neurons, so as to make them communicate within the same drop, or interactions between the various types of cells which make up the skin, in order to mimic the behavior thereof on a cellular scale.

It is also possible to reconstitute a cellular tissue intended to mimic the behavior of the epidermis by culturing together, within the same drop, keratinocytes on a layer of collagen. It is also possible to culture together skin stem cells in the presence of hair follicle cells in order to study their interactions.

For example, it is possible to use the transfection of reagents into a first type of cells in order to trigger a cellular reaction, such as the production of a recombinant protein, and then to react this first cell population with a cell population of another type by fusion with another drop.

According to a variant of the invention illustrated in figure 8, it is also possible to envision that the support is equipped with separation means that make it possible to separate two different cell types but that permit the passage of small molecules between these cells. Such a separation means is intended to mimic a biological barrier, such as, for example, the barrier that exists between the blood and cervical cells. Such separation means are advantageously arranged on the reception means on the support. In order to use them, it is envisioned to deposit an aqueous drop comprising at least one cell of a first type on one side of the separation means and an aqueous drop comprising at least one cell of a second type on the other side of the separation means. The fusion of the drops on either side of the separation means allows communication between the cells by means of molecules capable of diffusing through the separation means. This

communication can then be studied by any means, in particular by the addition of reagents in the form of an aqueous drop before or after the fusion of the cell drops. Through the automated transfection of these drops, it is possible to analyze the biological role of the factors transfected in a biological multilayer.

The separation means that can be used according to this variant of the invention are artificial membranes such as, for example, a nitrocellulose filter, silicon pierced with nano-holes, blotting paper, a cloth filter; the use of a solid gel, such as an agarose, collagen or gelatin gel, can also be envisioned.

The device and the method according to the invention make it possible to automate the expression of recombinant proteins obtained by the entry of coding DNA into the cells, to carry out the screening of nucleic acid molecules intended to modify (to block or, on the other hand, to increase) gene expression in the cells, and to search for promoter genomic sequences. This invention also makes it possible to study the interactions between cells of different types, this interaction being triggered by the mixing of the drops. The device and the method according to the invention make it possible to obtain an overall view of the biological effects of the reaction of molecules of all sorts with cells, and in particular of the automated entry of molecules of all sorts into cells.

Advantageously, the overall detection of the cell phenotypes engendered by the entry of the molecules into the cells will be carried out by means of labeled molecules i.e. of molecules which can be detected without affecting the integrity of the medium which contains them. This involves in particular fluorescent or radioactive labels or any other labeling means known to those skilled in the art.

One of the advantages of the method and of the device according to the invention lies in the fact that all the steps of transfection and of manipulation of the cellular interactions are carried out in a liquid phase which promotes the cell culture in nutritive medium and the enzymatic reactions.

In general, the method and the device according to the invention have the following advantages:

- an improvement in transfection efficiency, compared with transfection conventionally carried out in culture wells;

- several cell types can be tested on the same support during the same reaction sequence;

- several molecular types can be tested on the same support during the same reaction sequence;

- the reagents can be obtained directly in the drop before fusion;

- all the reagents are prepared independently and in parallel with the preparation of the cell cultures.

Among the applications of the device and of the method according to the invention, mention may in particular be made of the search for sequences with antisense activity. The use of small DNA or RNA sequences for blocking the synthesis of a protein in cells and in animals is known through Dean *et al.*, Current Opinion in Biotechnology, 12, 622 (2001). This gene therapy has been used to test novel antiviral agents in humans. However, this biotechnological approach has failed a great many times, the use of one or more oligonucleotides not making it possible to block the expression of a protein. In these assays, in the absence of suitable screening means, the sequences of

the oligonucleotides used were chosen slightly randomly, from the accessible genomic sequences. The oligonucleotides used did not have sufficient affinity for the target RNA and did not make it possible to block translation thereof in the cells. Since synthetic DNA molecules are toxic for eukaryotic cells, the desire is to use them in minimal amounts. To block the expression of a target gene in the cell, it is necessary to intervene at four levels:

- find the optimal binding position on the RNA, which position generally corresponds to a less folded portion in the quaternary structure of the RNA;

- find oligonucleotide sequences that have high affinity for the RNA;

- find oligonucleotide sequences capable of penetrating into a eukaryotic cell;

- keep the oligonucleotides nondegraded in the cell for several days.

The first two steps can be carried out using a conventional oligonucleotide chip on which is tested the hybridization of a family of oligonucleotides with target RNA made fluorescent (reference may, for example, be made to the studies of Olejnik *et al.*, NAR, 26, 3572 (1998)). The method according to the invention will make it possible to test the penetration of the oligonucleotides into the cell and their stability.

In order to demonstrate the optimal sequence for reducing the expression of a target protein in a cell, modified oligonucleotides, such as for example phosphorothioate derivatives, will be used, this modification conferring on the oligonucleotide concerned a nuclease resistance of several days.

The antisense molecules may also consist of RNA duplexes, called interfering RNAs (iRNAs), which hybridize to the messenger RNAs, forming RNA triple helices (for this approach, reference may be made to Elbashir *et al.*, Nature, 411, 494-498 (2001)). The method and the device according to the invention also make it possible to screen long (plasmid) and short (synthetic) iRNAs.

Another application of the method and of the device according to the invention concerns the automated production of recombinant proteins in drops. Automatic transfection should make it possible to test the expression of various coding DNA fragments and also that of various mutants of this same DNA. The automated expression of recombinant proteins on the devices according to the invention may be an alternative to the protein chip. It is then no longer necessary to produce the proteins, to purify them and to attach them to a solid support, the proteins are produced *de novo* on the device.

Another application of the method and of the device of the invention is the preparation and the screening of siRNA. siRNAs (small interfering RNAs) are double-stranded RNA molecules capable of specifically and effectively blocking gene expression in cells in culture and in animals. siRNAs were voted "molecules of the year 2002" by the magazine Science: these molecules could allow in particular innovative therapies in cancerology and in virology. They have also already made it possible to characterize gene function that was up until now unknown (T. Tuschl, Nature Biotechnology, vol. 20, May 2002, p. 446-448).

Four approaches have been recorded for producing these siRNAs. The first uses chemistry, each of the two siRNA strands are produced in a nucleic acid synthesizer and are then associated after synthesis (synthetic molecule

produced from phosphoramidite RNA described in: S.M.Elbaschir *et al.*, Nature, 411, May 24, 2001, 494-498). The second uses an RNA polymerase *in vitro*, each strand of the siRNA is produced in complementary fashion relative to a DNA strand, and the two strands are then associated after synthesis (example: pSilencer commercial kit sold by the company Ambion and HiScrib commercial kit sold by the company NEB Biolabs). The third uses *in vivo* synthesis in eukaryotic cells or in bacteria, the two strands are produced using the RNA polymerases present *in vivo*. The fourth uses single-stranded or double-stranded long RNAs (produced *in vitro* or *in vivo*) which will be converted into small molecules (siRNAs) by the nucleases of the transfected cells (R. Agami, Current Opinion in Chemical Biology, 2002, 6, 829-834).

In the present invention, it is the second approach which can be applied, that which endeavors to produce siRNAs from DNA molecules contained in a tube. This approach is in particular used since it is less expensive than that which consists in producing the siRNAs chemically. It has also been reported that the structure of siRNAs synthesized *in vitro* is different from that of siRNAs obtained chemically; it makes it possible to obtain a greater affinity in the case of the hybridization of siRNAs obtained *in vitro*, with the target RNAs of the transfected cells. Thus, the blocking of the gene expression is better with siRNAs obtained *in vitro* than with chemical siRNAs: better efficiency and no need for such high siRNA concentrations.

This variant of the invention is based on a method of producing siRNAs on a solid substrate. The present invention proposes to produce siRNAs in a drop by means of two DNA matrices containing a promoter sequence for an RNA polymerase (of SP6 or T7 type, for example) and using this drop format for transfecting, by drop

fusion, the double-stranded siRNA molecules into cells cultured in a drop or on a solid substrate. The DNA matrices can be covalently or noncovalently attached to the solid substrate.

In order to optimize the blocking of the expression of a gene, it is necessary to test several siRNA sequences along the gene (at least ten or so) and considerable interest is turning toward the systematic screening of several genes (for example more than 5600 genes blocked in the earthworm: S.S.Lee et al., Nature Genetics, 33, January 2003, 40-48).

Certain siRNA sequence qualities have already been proposed regarding the percentage of GC nucleotides for example, or the Tm of the corresponding oligonucleotide, or else the size of the molecule (21mer). However, nothing replaces experimentation; screening is necessary not only in order to find the optimal sequence for blocking the expression of a gene, but also for determining its concentration and also its action time in the cell culture.

In order to perform these various phenotypic screenings, it is possible to use plastic wells (96-well or 384 format); however, we have shown that transfection efficiency is better in cell cultures in drops (a 5 times lower concentration of siRNA was used to obtain the same efficiency of transfection and of blocking of the expression of a gene in cell cultures in drops, rather than in cell cultures in wells). In addition, the phenotypic analysis of the blocking of the expression of a gene is very relevant when the cells have been cultured in a drop on a glass slide: the cells, after they have been transfected, are attached to the glass slide in small distinct piles, and the gene expression levels are revealed by fluorescent immunocytochemistry and analyzed directly by scanner (direct detection of the number of photons

emitted by the cells labeled with antibodies).

Potential applications:

- Characterization of gene function (in particular in the case of genes that are "inaccessible" by KO of transgenic mice (the KO mice died before birth).

- Characterization of the function of several genes in several types of cells: high throughput screening necessary. This is the case of the *in vivo* analysis of gene deletion.

- Cancerology: analysis of the role of genes in angiogenesis and also in the propagation of certain cancers. In addition, blocking the genes essential to cell repair will make it possible to render certain cells chemosensitive. The chemosensitivity can be analyzed on a chip.

- Virology: Propagation of the HIV virus has been blocked *in vivo* through using siRNA (N.S.Lee *et al.*, Nature biotechnology, Vol. 19, May 2002, 500-505).

EXAMPLES

Materials and methods used in the examples:

Detection

The detection of transfection, with a fluorescent molecule, is carried out in two steps:

- by means of an overall view of the fluorescent spots,

- by analysis of the individual spots so as to create a complex image.

The overall view of the fluorescence is determined by

biparametric detection:

- of propidium iodide fluorescence = reflects the presence of a cell,

- of green fluorescent protein (GFP) fluorescence = green fluorescent label reflects expression of the GFP protein.

The image obtained is then analyzed by means of signal quantification systems which make it possible to combine the fluorescences of series of measurement points.

- Analysis of the drop fluorescence: the cells can be analyzed while alive in drops under the layer of oil. For these experiments, we used an Olympus® BX 51M microscope and a Leica® confocal microscope.

- Analysis of the fluorescence of the fixed cells: after fixing of the cells with PFA, or paraformaldehyde (see application No. 1), the glass slide containing the recombinant cells is used as a histology slide. It is possible to carry out radioactive labeling and fluorescent labeling experiments (see application No. 5.2). The total fluorescence of the slide can be analyzed using a conventional microscope. We also used a scanner conventionally used in DNA chip experiments (scanner: GenePix 4000B, sold by the company Axon Instrument). The precision in this type of device is 5 µm, it is therefore possible to visualize a cell using about 10 pixels.

Figure 4 illustrates an example of application of the device according to the invention: the expression of a recombinant protein in a suspension of glial cells and of the activation of a suspension of neurons.

The following are injected onto a glass slide (support

S) in a container containing a light mineral oil (film F) sold by the company Sigma:

- a drop of cells in suspension containing, in aqueous medium (a few nanoliters), about a hundred glial cells;

- an aqueous drop containing DNA in the form of a calcium phosphate salt (a few picomoles);

- a drop of neuronal cells in suspension in water.

The first two drops are first of all made to fuse by mechanical displacement of the two drops using the end of a pipette, so as to obtain drop G_1 of transfected cells. The glial cell thus expresses a recombinant protein. This drop G_1 is then fused with the drop G_2 containing the neurons in suspension. These neurons are then activated.

I. Transfection of oligonucleotides, screening of sequences capable of blocking the expression of specific genes

In this example, we sought to select oligonucleotide sequences capable of blocking the expression of a gene of interest, called target in this example. To measure the antisense activity of these oligonucleotides, we used stable cell lines expressing the target protein as a fusion with the E-GFP variant (green fluorescent protein type E, sold by the company Clontech). These lines are precious tools for evaluating the performance levels of the various oligonucleotides: the antisense activity is measured by means of a decrease in the fluorescence of the reporter proteins. In order to find an oligonucleotide which has exceptional antisense activity, it is essential to screen at least 50 oligonucleotides having different sequences, for the same gene. In the course of this screening in the cells, we sought in particular the oligonucleotide

which has the greatest affinity for the target RNA and the greatest ability to penetrate into the cell.

Experiments carried out with the device according to the invention:

Calcium phosphate transfection was chosen since it is very efficient: using oligonucleotides labeled with cy5 (cyanin 5), these oligonucleotides being sold by the company Eurogentec, 70% of HEK 293 (human embryonic kidney) cells and 80% of COS (Chinese ovary sarcoma) cells become fluorescent after one day of culture (fluorescence measured by flow cytometry). Other transfection methods involving the formation of droplets of lipid around the DNA can also be used.

Experiment 1a: Transfection by drop fusion

For a target gene, 50 oligonucleotides having different sequences are chosen. We endeavored to block the expression of the beta 2 subunit of casein kinase. The oligonucleotides are synthesized in the form of residues 18 to 21 nucleotides long, and contain phosphorothioate bonds capable of limiting their degradation by nucleases. Each of these oligonucleotides is precipitated in calcium phosphate form (conventional reaction: 1 µl of oligonucleotide resuspended in water at 1 mM is mixed with 100 µl of 0.25M calcium chloride and with 100 µl of HBS buffer (Chen and Okayama, Biotechniques 1988, 6, 632-638)). To perform the transfection in a plastic dish containing 1 ml of mineral oil, a 1 µl drop of each of these 50 precipitates is fused with a 10 µl drop of cells in their culture medium (DMEM sold by the company Gibco) (approximately 5000 3T3 fibroblast cells). The drops are placed at the bottom of the dish on the plastic support under the oil.

The antisense activity is measured after 2 days of

culture, by means of a decrease in the fluorescence of the GFP expressed in tandem with the target protein for which blocking of the expression is sought. The fluorescence of the 50 drops is observed simultaneously under microscope. This experiment is carried out several times in parallel in order to be sure that the results are relevant. It is important to carry out these 50 tests in parallel in order to compare the antisense activity of each of the oligonucleotides. In order to observe the decrease in fluorescence of the target protein, it is possible either to observe the transfected cells under a microscope while they are alive, or to fix the cells with paraformaldehyde (conventionally 4% of PFA). To fix the cells, the paraformaldehyde is added, at equal volume, to the cell drop for 10 minutes and then the drops + oil mixture is rinsed twice with PBS.

Experiment 1b: Photocleavage of the oligonucleotides from the cell chip and transfection

This experiment is illustrated in figure 3.

A conventional DNA chip was used, and the transfection is obtained after detachment of the oligonucleotides from the solid support. The cells are cultured in the vicinity of the oligonucleotide deposits on the glass slide.

The oligonucleotides (sold by the company Eurogentec) are attached, via their 5′ amino end, to a silanized slide (for example a Surmodics slide sold by the company Motorola). The DNA in the deposits is complexed with calcium phosphate salts (according to the same principle of DNA precipitation as that described in example 1a) and kept wet on the slide. The adherent 3T3 cells are then deposited in a drop at the surface of the oligonucleotide spots, and the entire combination is kept under 1 ml of mineral oil for one day in a cell

incubator: the drops can be formed at the surface of the DNA deposits or else formed using a composite surface (hydrophilic and hydrophobic, ProLabo slide described in application No. 5). Detachment of the oligonucleotides complexed with calcium phosphate and located under the cell cultures in a drop is obtained, as illustrated in figure 5, by illumination of the slide with UV radiation at 365 nm, which makes it possible to cleave the photocleavable bond introduced in the 5' position of the oligonucleotides (in the position 3' of the amino site).

Example of an oligonucleotide attached and then detached by photocleavage and then finally transfected into adjacent HEK 293 cells:

Name	**Sequence**	**Composition**	**Modification**
huGAPDH203F_PC_cy5	SEQ ID NO:1 AACGACCACTTTGTCAAGCT	20 mer + PC site + Cy5 site + amine in the 5' position	PC in the 5' position - Cy5 in the 3' position (dt)

II <u>Transfection of coding DNA and expression of recombinant proteins</u>

In this example, we focused on the expression of a family of proteins in eukaryotic cells. For the moment, in molecular biology, the expression of recombinant proteins is obtained well by well, by transfection of a coding DNA on a cell layer. By means of paralleled transfection, we will simultaneously obtain the expression of several DNAs encoding several proteins. This device makes it possible to test several constructs in vectors for expression of one or more genes.

<u>Experiments carried out with the device according to</u>

the invention:

Application 2a: Expression of recombinant proteins in one cell type

Expression of a DNA family encoding human kinesins by means of the device according to the invention:

Kinesins form a family of proteins having similar biochemical properties; these are microtubule motor proteins. These proteins are in all eukaryotic cells. They make it possible to convert ATP hydrolysis into mechanical energy and play a fundamental role in the transport of organelles, of mRNAs and of protein complexes along the microtubules. They can move on the positive side of microtubules (N-kinesins) or on the negative side (C-kinesins). They also participate in chromosomal movements during mitosis and meiosis and play an important role in cell division. Reference may in particular be made to the following publications:

Ref. 1: Compton, D.A. (1999). New tools for the mitotic toolbox. Science 286, 913-914. Miki, H., Setoy, M. Kaneshira, K., Hirokawa, N. (2001). Proc. Natl. Acad. Sci. 98, 7004-7011.

Ref. 2: Wade, R.H., Kozielski, F. (2000). Structural links to kinesin directionality and movement. Nature Structural Biology 7, 456-460.

Ref. 3: Kozielski, F., Svergun, D.I., Zaccai, G., Wade, R.H., Koch, M. (2001). The overall conformation of conventional kinesins studied by small-angle x-ray and neutron-scattering. J. Biol. Chem. 276, 1267-1275.

With the device according to the invention, we obtained the expression of about twenty DNAs encoding these kinesins, using expression plasmids for expressing the protein of interest and GFP in tandem (pcDNA3.1/CT-GFP-

TOPO, sold by the company In Vitrogen). The transfection was carried out as in application 1a: fusion of the drops is obtained under 1 ml of oil, 10 ng of calcium phosphate-precipitated plasmid are brought into contact with a 10 µl drop containing approximately 5000 HEK 293 cells (human embryonic kidney cells). After 1 day of transfection, the drops of HEK 293 cells are fluorescent through expression of the GFP protein in the cells. It was possible to observe these cell drops under oil on a confocal microscope and to precisely localize the cellular compartmentalization for each of the fluorescent kinesins expressed in this device.

Application 2b: Expression of recombinant proteins in a device according to the invention using several cell types.

Recombinant expression of factor Pax6 by glial cells: 10 ng of calcium phosphate-precipitated (see precipitation of DNA with calcium phosphate in application 1a) plasmid containing in tandem the genes encoding factor Pax6 and GFP (pcDNA3.1/CT-GFP-TOPO, sold by the company InVitrogen) are brought into contact with a 10 µl drop containing approximately 5000 glial cells (post-natal radial glial cells from cortex, 2 divisions). After 1 day of culture, the drop G1 of glial cells is fused with another drop G2 of 10 µl containing 5000 cortical cells (cortical primary culture isolated from post-natal cerebral cortex). The recombinant glial cells of G1 expressing factor Pax6 are, for example, capable of inducing neurogenesis of the astrocytic cells contained in G2.

This application is important for the screening of novel medicinal products with neurotrophic potential, for example in the search for compounds capable of regenerating dopaminergic neurons in brains impaired by Parkinson's disease.

Reference may, for example, be made to the following publication:

Ref. 4: Heins et al., Glial cells generate neurons: the role of the transcription factor Pax6, Nature Neuroscience (2002) 5, 308-315

III - Transfection with viruses or prions in confined medium

The system according to the invention can be readily confined: the formation of the drops can be carried out mechanically without intervention of a user, and these drops can be kept away from contaminants by being maintained under a layer of oil for several days. Viral transfection can be obtained in two ways:

- by fusion of a drop of eukaryotic cells and of a drop of virus

- by contamination of cells with cell samples "at risk".

It is possible to produce the virus or the pathogenic agent (amplification and encapsulation), or to detect it (biological concentration and antigen-antibody reaction), in the drops.

IV - Demonstration of a promoter genomic sequence

Promoter sequences, a relatively unknown part of the genome:

Reporter gene technology, and more precisely "promoter region-reporter activity" construct technology, has been widely used for characterizing the regulatory regions upstream of genes. Microtechnology today makes it possible to considerably increase the number of promoters studied. The transcriptional regulation of

these regions can be observed in real time through the use of a reporter gene encoding GFP (green fluorescent protein). The fluorescence of this protein can be observed without having to lyze the cells. The variations in fluorescence will allow us to study the kinetics of the transcriptional regulation of a large number of promoters in parallel and in real time.

Experiments carried out with the device according to the invention:

The reporter activity of known genes induced by ionizing radiation (for example the p53 and c-myc genes) was used to validate the chip and the experimental model. The sequences of interest upstream of the p53 and *c-myc* genes were amplified and then cloned upstream of the GFP gene in the vector phrGFP (Genentech). The transfection was carried out as in application 1a: fusion of the drops is obtained under oil, 10 ng of calcium phosphate-precipitated plasmid (phrGFP containing the DNA promoter sequences) are brought into contact with a 10 µl drop containing approximately 5000 human keratinocyte cells. By virtue of its location in the skin, the keratinocyte represents one of the cell types most exposed to irradiation *in vivo*. After 1 day of transfection, the drops of keratinocytes are fluorescent due to expression of the GFP protein in the cells. The GFP reporter activity is measured by overall reading of fluorescence by microscopy coupled to a CCD (charge coupled device) camera.

V - Screening of novel medicinal products in drops:

General comment: In the pharmaceutical industry, screening has been carried out, for the last ten years, on recombinant protein targets. In several examples (such as glutamate receptors), it was noticed that the recombinant receptor present in the isolated plasma membrane was not at all in its biological conformation; the receptors need in particular to be coexpressed with chaperone proteins which are present in the synapses. Reference may, for example, be made to the following publication:

Ref. 5: Ohnuma et al. Gene expression of PSD95 in prefrontal cortex and hippocampus in schizophrenia. Neuroreport. (2000) 11 (14):3133-7.

In order to obtain a result that is more precise and closer to *in vivo* behavior, the screening of a set of chemical compounds is now envisioned on living cells. In this case, the screening is carried out using a dynamic test during which the cells are kept alive. Reference example:

Ref. 6: Fluorescent indicators for imaging protein phosphorylation in single living cells, Sato et al., Nature Biotech (2002) 20, 287-294).

Study model: Preparation of the cell drops

This experiment is illustrated in figure 6. The living cells are deposited in homogeneous drops on a solid support. The preparation of this cellular device can be facilitated by using a composite surface (hydrophilic and hydrophobic), a glass slide sold by the company ProLabo, covered with a Teflon® film and containing hydrophilic circular wells 3 mm in diameter. A large number of homogeneous drops can be produced by simply dipping the support in a cell suspension. The drops are

then covered with a layer of mineral oil in order to prevent them drying out and to promote cell survival until the screening assay. The cell drops can be conserved on this type of support for several days in an incubator intended for cell culture. To screen n different molecules, n drops of test compounds are deposited individually on the cell drops previously formed.

It is also possible to envision that chemical compounds are grafted onto the surface of the support (as for the oligonucleotides, in application 1b) and then detached together (for example by UV illumination). The insertion of a photocleavable site into the molecules can be obtained by combinatorial chemistry.

A simple dynamic screening test (example: FRET = fluorescence resonance by electron transfer) can be used to demonstrate the properties of some of these compounds.

5.1. Screening example: measurement of the activity of the adrenergic receptor:

Reference may be made to the following publication: *Ref. 7: Ghanouni et al., Agonist induces conformational changes in the G protein coupling domain of the beta2 adrenergic receptor, PNAS (2001) 98, 11, 5997-6002.*

It is possible to attach a fluorescein to one of the cysteines (cys265) of the beta2 adrenergic receptor in order to render this protein fluorescent when it is inserted into the cell membrane. In order to test the adrenergic properties of a novel potential medicinal product, a 100 nl drop containing the molecule in solution is brought into contact, under oil, with a 1 µl drop containing 500 recombinant cells, in their culture medium, expressing the recombinant and mutated adrenergic receptor. The induction of a conformational

change in the receptor by an agonist is observed, under the microscope, through a decrease in the intensity of fluorescence of the fluorescein contained in the adrenergic receptor.

5.2. Screening example: immunocytochemistry on recombinant cells:

After transfection, the recombinant cells can be fixed on the support using PFA (see application No. 1). In the case of the recombinant expression of the beta 2 subunit of caseine kinase with the device according to the invention, we used an antibody to reveal the expression of the recombinant protein in the 3T3 cells (antibody sold by the company Upstate Biotechnology: Rabbit polyclonal anti-CK2 beta antibody). Reference may be made to the publication *Ref. 8: Alexandre E. Escargueil et al., Mitotic Phosphorylation of DNA Topoisomerase II by Protein Kinase CK2 Creates the MPM-2 Phosphoepitope on Ser-1469, J. Biol. Chem. (2000), 275, Issue 44, 34710-347183.*

VI - Variant of the molecule chip:

Figure 7 illustrates this variant: drops of cells in a culture medium are deposited in the form of a matrix on a Teflon support. A PDMS cover, onto which are grafted organic molecules of a screening library which comprise cavities having a volume substantially equal to that of the drops, is placed on the support. The spacing between the drops was designed so as to correspond exactly between the support and the cover. The molecules are detached from the cover by any appropriate treatment, so as to transfect cells.

VII - Displacement of drops in an electric field:

The displacement of drops on a support, the surface tension of which varies with the application of an

electric field, was tested: on the support tested, which is hydrophobic in nature with the exception of hydrophilic blocks, the surface tension decreases with the intensity of the field; the surface becomes less hydrophobic, or even hydrophilic. The control and the movement of the electric field make it possible to displace the drops of liquid on this surface. The movement of drops of cells was carried out in an electric field of 1000 V (a drop of 5 µl containing a Hela cell suspension at 10^6 cells/ml, a drop of 5 µl: trypan blue in PBS (1/5)).

Trypan blue is a cell viability marker. This trypan blue and cell mixture reaction made it possible to verify that the electric field had not killed the cells, since the % of stained cells did not exceed 2%.

VIII - Automated transfection:

The performing of transfections on chips containing cells in a drop, carried out manually on commercial slides, can be transferred onto an automated device for producing microbatteries, and can thus make it possible to carry out a specific method of automated DNA transfection. For this, three series of successive deposits of three drops are effected on the same chip, and therefore on the same block of the commercial slides; the drops will fuse to allow the reagents and the cells to mix.

Experiment:

1. Sample preparation:

A plate containing 96 wells containing the various samples to be deposited: the DNA solutions, the transfection medium, the cell media, is prepared.

2. Depositing of the DNAs (single-stranded

oligonucleotides, PCR products or plasmids) to be transfected into the cells:

The robotic device takes, from the 96-well plate, 2 µl of a solution containing one of the DNAs and it then deposits a 10 nl drop on one of the blocks of the slide, and so on, for the various DNAs. We therefore deposited 10 nl of oligonucleotide at a concentration of 10 nM (oligo: 5'SEQ ID NO:2 CGGAGGCGATGGTGTTGGA 3' - 20mer 5'-labeled with CY3) and 10 nl of a solution of plasmid encoding the GFP protein (pEGFP-C1, Clonetech) at a concentration of 1 mg/ml. Figure 9 is a photograph representing the plate on which the first three drops were deposited.

3. Depositing of the transfection solution on the blocks of DNA to be transfected:

For this, a drop of 0.2 µl of "siPORT" (Ambion), diluted to 1/20 in PBS (supplier's protocol), is deposited on each of the blocks and mixed with the DNA solutions deposited beforehand.

4. Depositing of the cells:

We deposited a 1 µl drop containing 10^3 HeLa cells (initial concentration of 10^6 cells/ml) on each of the blocks already containing the DNAs and the siPORT.

After the cells had been deposited, the slides are placed in a Petri dish containing PBS (to prevent evaporation of the cell medium). They are then transferred into a cell incubator (37°C, 5% CO_2) for 48 hours so as to allow expression of the EGFP protein.

After this culturing step, the cells are fixed in a solution of paraformaldehyde (4% in PBS) for 20 minutes, and then rinsed twice with PBS. The slides are then mounted in PBS and the fluorescence is then

observed under a microscope.

Observation of the cells under an Olympus BX52 microscope shows a diffuse fluorescence in the cytoplasm for the transfection with the oligo Cy3. the fluorescence is first more intense in the nucleus and then it diffuses into the cytoplasm, for the cells transfected with GFP. The negative controls (no cells or no DNA) show no fluorescence.